GAMMA-INTERFERON ACTIVITY MODULATING AGENT
[Agent Modulateur de l'Activité de l'Interféron-Gamma]

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The invention relates to a γ-interferon activity modulating agent. /1

γ-Interferon is a cytokinine which plays a very important role in the immune response. It is known that γ-interferon intervenes in the regulation of the induction of the cytotoxic T lymphocytes, which increases the activity of the NK killer cells, promotes the function of presentation of the antigens by the macrophages, and is used notably as an antitumor and antiviral drug.

The development of a therapy based on the use of γ-interferon poses, however, difficult technical problems, notably because of its short in vivo half life and its substantially local action, which requires the development of special administration procedures; see, for example, Yoshihiko Watanabe et al., P.N.A.S. USA, 86, 9456-9460 (1989); J. du Plessis et al., Antiviral Research, 18, 259-265 (1992); and C. Boutin et al., Cancer, 74, 2460-2467 (1994).

It is known that γ-interferon binds to the extracellular matrix of the conjunctive tissue, and more particularly to the proteoglycanes with heparane sulfate. The C-terminal part of γ-interferon comprises a peptide sequence 125-131, which is rich in units derived from basic amino acids, which intervene primarily in the binding affinity of γ-interferon for heparane sulfate; see, for example, H. Lortat-Jacob et al., C.R. Acad. Sci., Paris, t.311, series III, 143-147 (1990); FEBS Lett., 280, 152-154 (1991); and J. Clin. Invest., 87, 878-883 (1991). In addition, it has been suggested that the binding to heparane sulfate stabilizes and activates γ-interferon, and some hypotheses have been proposed to attempt to explain these phenomena; H. Lortat-Jacob and J.-A. Grimaud, Cell. Mol. Biol., 37, 253-260 (1991).

Attempts to identify the sites of heparane sulfate that are responsible for the affinity to γ-interferon have been carried out in preliminary studies which led to the hypothesis that the binding with γ-interferon occurs by carboxylated groups and not by the N-sulfated groups, which are present in a

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domain of heparane sulfate called "heparine type domain," see H. Lortat-Jacob and J.-A. Grimaud, Biochimica et Biophysica Acta, 1117, 126-130 (1992).

It has now been discovered that in fact the high affinity of heparane sulfate for γ-interferon results from the pressure, in heparane sulfate, of two domains which contain sulfated groupings and which each bind to the C-terminal part of one of the chains of the γ-interferon present in the form of a homodimer. The two binding domains are connected, in heparane sulfate, by a weakly sulfated sucrose chain having a length such that the groups involved in the binding with the C-terminal part can be located respectively opposite their binding site on the homodimer. Thus, the organization of these domains confers to heparane sulfate a strong affinity for γ-interferon.

This discovery has made it possible to design an agent that notably presents a capacity to potentiate γ-interferon, notably facilitating the transport of γ-interferon into the systemic circulation and into the tissues, by preventing the γ-interferon from being captured by the extracellular matrix of the conjunctive tissue. Thanks to the potentiating agent according to the invention, one can consider, for the first time, an action of the interferon by the systemic route, rather than simply a local action. In addition, the potentiating agent of the invention increases the half-life of γ-interferon in the organism while at the same time preventing or minimizing degradation, and promoting its activation, as will be shown in the experimental part below. Moreover, the transport action of γ-interferon by the agent of the invention also applies to endogenous γ-interferon, and thus it can modify, reduce or eliminate the local action of the latter. For example, it is known that γ-interferon introduces the expression of class II antigens and the expression of adhesion molecules (ICAM) of the T lymphocytes, by the endothelial cells, and can thus participate notably in the rejection of organ grafts. The agent of the invention can have an antagonistic effect on this action of γ-interferon, and thus has an immunosuppressant activity. Thus, the agent of the invention is capable of modulating the activity of endogenous and/or exogenous γ-interferon.

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Consequently, such a modulating agent can have a potentiating effect or an antagonistic effect, depending on the cases.

It is recalled that the acronym ICAM denotes intercellular cell adhesion molecules (Intercellular Cell Adhesion Molecule).

The invention thus relates to the use as a γ-interferon activity modulating agent of a compound comprising a grouping having the formula A-X-B, in which A and B represent independently an oligosaccharide group that carries a sufficient anionic charge to confer to said oligosaccharide group an affinity for a portion of the C-terminal end of human γ-interferon containing the peptide sequence 125-131, and X is a length spacer arm sufficient to allow the groups A and B to be connected by affinity binding each to one of the C-terminal ends of a homodimer of γ-interferon.

In the formula A-X-B, A and B are connected to the spacer arm by covalent bonds.

It is recalled that the peptide sequence 125-131 of the C-terminal end of human γ-interferon is as follows (see, for example, Rinderknecht et al., J. Biol. Chem., 259, 6790-6797 (1984)):

Lys Thr Gly Lys Arg Lys Arg.

The invention concerns particularly the utilization of the compounds A-X-B as an active ingredient in the preparation of a drug intended to modulate the activity of γ-interferon.

To better understand the experimental discoveries, which are the basis of the invention, some physicochemical data must be recalled beforehand regarding γ-interferon, and the polysaccharides of the heparane sulfate and heparine type.

It is known that heparin and heparane sulfate are part of the family of the glycosaminoglycanes, which are products that have repeated disaccharide osamines/uronic acid units.

The structure of heparane sulfate from human fibroblasts has been studied notably by J. E. Turnbull and J. T. Gallagher, Biochem. J., 273, 553-559 (1991). These authors have shown that the

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depolymerization of heparane sulfate by heparinase cleaves specifically strongly sulfated disaccharides of the structure α-glucosamine (N-sulfated and optionally 6-sulfated) - (2-sulfated) 1,4-iduronic acid, and that heparane sulfate contains approximately 10% bonds that can be cleaved by this enzyme. The products of the depolymerization with heparinase are oligosaccharides having an approximate mean molecular weight of 10 kDa. These oligosaccharides, which are resistant to heparinase, are very sensitive to the depolymerization by heparitinase, which cleaves heparane sulfate in areas with low sulfatation, where the predominant disaccharides are of the α-glucosamine N-acetyl type (or occasionally of the N-sulfated type) - 1,4-glucuronic acid. The same authors have also shown that the sites of cleavage by heparinase are regrouped in domains having average lengths of 4-7 disaccharides. Thus, heparane sulfate has a polymer structure consisting of successive arrangements of highly sulfated and weakly sulfated domains.

In heparine (see, for example, J. T. Gallagher and A. Walker, Biochem. J. <u>230</u>, 665-674, 1985), the osamine can also be the N-acetylated or N-sulfated glucosamine, and the uronic acid is primarily, optionally 2-sulfated, L-iduronic acid. Its structure is thus similar to that of heparane sulfate, differing from the latter only in the degree of sulfatation and the degree of epimerization of the glucuronic acid to iduronic acid. Consequently, heparin presents low sensitivity to the action of heparitinase (which cuts the glycosaminoglycanes at the level of a glucuronic acid), but it is strongly depolymerized by heparinase (which cuts at the level of a 2-sulfated uduronic acid).

Some embodiments of the invention will now be described in greater detail.

The modulating agent according to the invention contains terminal groups A and B, which may or may not be similar, and which consist of any oligosaccharide carrying a sufficient anionic charge to allow binding with the C-terminal part of γ-interferon, by anion-cation type affinity.

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The oligosaccharide groups can contain notably sulfate and/or phosphate groupings, with a sulfatation and/or phosphatation percentage which is sufficient to confer affinity for the C-terminal part of γ-interferon.

In particular, the oligosaccharide groups A and B contain units that are derived from an N-sulfated osamine, or units derived from sulfated uronic acid, or any other units that carry anionic groups in a sufficient quantity to confer to said groups A and B an affinity for γ-interferon, and, in particular, for a part of the C-terminal end of γ-interferon.

Among the oligosaccharide groups that constitute the ends A and B, the oligosaccharide groups that are cited notably contain alternating disaccharide units derived from an osamine or from uronic acid, as in the glycosaminoglycans.

These oligosaccharide groups A and B can be notably depolymerization fragments that can be obtained by reacting heparitinase with heparane sulfate. Other depolymerization products can also be present, which may optionally be subjected to a substitution reaction with substituents that carry an anionic group (for example, sulfate or phosphate), according to the known methods, if the starting product does not have a sufficient anionic charge.

The oligosaccharide groups A and B can contain, for example, 6-14, particularly 6-10 saccharide units.

The spacer arm represented by X in the above-mentioned formula of the potentiating agent according to the invention can be any polymer (for example, polyoside or polyglycol) having a sufficient length to allow the terminal groups A and B of binding each to one of the C-terminal ends of a homodimer of γ-interferon. The expression polyglycol denotes conventionally a poly(alkyleneoxy) arm.

The spacer arm can also be derived from a polyester, such as, a poly(lactic acid) or a poly(glycolic acid), whose ends are bound to the groupings A and B by ester or amide bonds, for example.

In the experimental part below, it is shown how this sufficient length can be determined by simple routine experiments. Naturally, the modulating agent can be present in the form of a Y-A-X-B-Z compound in which Y and Z, which may be similar or different, are polymer or oligomer groupings that are analogous notably to the groupings X, A or B (see Example II of the experimental part below).

More generally, each one of the experiments described in Example II can be used to select the most appropriate modulating agents.

For example, in the case where the spacer arm is a polysaccharide, it can contain approximately 24-32 sucrose units.

When the spacer arm is a polyglycol, it can be, for example, an arm derived from a polyethylene glycol, that is a $-(CH_2CH_2O)_n-$ arm, where n is the number of ethoxy units.

In specific embodiments, the spacer arm is a polymer where at least some of its units can contain anionic groups, such as, sulfate, phosphate or carboxylic acid groupings. One can use notably as spacer arms fragments of the depolymerization of natural polyosides, such as, polydextran, cellulose, starch, carrageenan, fucoidan (Sigma), glycosaminoglycanes and their derivatives, notably their derivatives that carry anionic groupings.

These depolymerization fragments can be obtained by the known methods, notably by acidic or alkaline hydrolysis, by the action of ultrasound, by enzymatic digestion, or by a reaction of depolymerization by oxydo-reduction (called ORD) ("oxidative reductive depolymerization"); see, for example, Manssur Yalpani, "Polysaccharides-Syntheses, Modifications and Structure/Property Relations," Elsevier (1988).

The depolymerization fragments of appropriate length that are capable of constituting the X spacer arm are separated by the usual methods. If they contain no anionic groups, they may optionally be modified to introduce such anionic groups by the known methods. One can also use the products of the

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depolymerization of certain polymers that already contain anionic groups, for example, carboxymethyl or carboxyethyl cellulose, carboxymethyl or carboxyethyl starch, dextran sulfate, etc.

According to a specific embodiment, the spacer arm is a depolymerization fragment that can be obtained by reacting heparinase with heparane sulfate. For example, one uses depolymerization fragments having molecular weights of 6-7 kDa.

According to a special embodiment, the spacer arm can be N-acetylated heparin obtained by N-desulfatation followed by N-acylation; for the N-desulfatation and N-acylation methods, see particularly Methods in Carbohydrate Chemistry, Ac. Press., Vol. VIII, pp. 291-294 and the document PCT WO 92/22588.

The γ-interferon modulating agent can be prepared by reacting reactive derivatives that are precursors of the groups A and/or B, simultaneously or sequentially, with a reactive derivative that is a precursor of the spacer arm X, according to the usual methods that allow the formation of covalent bonds between A and X, and between B and X. It is within the competency of specialists to choose the derivatives that comprise the reactive groups of A, B or X and/or the protecting groups that allow the management of the reaction by protecting temporarily one of the reagent groups.

The reactive groups can naturally be present in the starting product or they can be introduced by the known methods.

For example, when the spacer arm is a polysaccharide compound, one can prepare the compound A-X-B by the known synthesis methods used in sugar chemistry; see notably "Carbohydrate Chemistry, John F. Kennedy Ed., Clarendon Press - Oxford (1988), 500-593; and Manssur Yalpani, "Polysaccharides-Syntheses, Modifications and Structure/Property Relations," Elsevier (1988), pp. 144-181. To obtain a polyglycol spacer arm, one can use, for example, a method analogous to the one described by P. Westerduin et al., Angew. Chem. Int. Ed Engl. 35(3), 331-333 (1996).

In some cases, one can prepare the potentiating agent of the invention by the partial depolymerization of a glycosaminoglycane (such as a heparane sulfate) which is appropriately protected: see, for example, the preparation of an IPD fragment in the experimental part below.

The potentiating agent can also consist of heparin or fragments of the depolymerization of heparin that are of appropriate size, and, in this particular case, there is no need to distinguish between the parts A, X and B (or A, X, B, Y and Z): the potentiating agent is produced directly by depolymerization and selection of the fragments of appropriate size. Some parts of the heparin molecule or of said fragments intervene in the bonding with γ-interferon and thus play the role of the parts A and B, while the middle area of said fragments, which has substantially the same structure as the end parts, plays the role of spacer arm. In the case where the modulating agent of the invention is based on heparin, it is generally desirable to modify the latter chemically to eliminate or decrease its anticoagulation activity.

The (optional) depolymerization of heparin (in the case where the starting heparin has a high molecular weight) can be carried out according to the known methods that allow the obtention of fragments of sufficient size. The isolation of the depolymerization fragments of appropriate size is carried out in a manner which in itself is known, for example, with a molecular mesh.

The modulating agent according to the invention can additionally be any biocompatible polymer (including sequenced copolymers) containing anionic groupings (sulfate, phosphate) in a sufficient quantity at at least two sites corresponding to A and B of the formula given above. One can use, in particular, polysaccharides which one can sulfate by the methods described in the literature (see, for example, Methods in Carbohydrate Chemistry Ac. Press, Vol. III, pp. 265-267 and Vol. VI, pp. 426-429).

One can also use commercial sulfated polysaccharides (see the Example II of the experimental part below).

The anionic groups present in the modulating agent according to the invention, or part of said groups, can be salted. One can notably use the alkaline or alkaline-earth salts, for example, the sodium salts.

The agent of the invention can be used as a drug. When it is used alone, it constitutes a drug intended to modulate the activity of endogenous γ-interferon. Such a drug is administered at doses that can be determined beforehand by routine experiments, notably as a function of the desired effect. These doses can range, for example, from 0.1 to 100 mg per individual and per day, notably 1-10 mg.

The modulating agent according to the invention can also make it possible to prepare a γ-interferon-based drug that can act by the systemic route. Such a drug contains thus, in combination, the modifying agent and γ-interferon, preferably in the amount of at least one mole of modulating agent per two moles of γ-interferon.

In this drug, the modulating agent prevents the capture of γ-interferon by the molecules of endogenous heparane sulfate, which are present, for example, in the extracellular matrix and thus allows its maintenance and its transport in the systemic circulation. In addition, the modulating agent protects the γ-interferon from degradations that can reduce or negate its activity, and it allows maintaining γ-interferon in its most active form until the time of its action on competent cells.

The drug of the invention is prepared according to the usual methods and it contains the vehicles and adjuvants required to present it in a form that can be administered notably by the intravenous, intramuscular or subcutaneous route, or by a local route (for example, cutaneous, intra-nasal or intra-bronchial).

When the modulating agent and the γ-interferon are used in combination they can be mixed beforehand in the drug, or they can be present in separate containers to be mixed at the time of use.

The drug of the invention, when it is used in combination with exogenous γ-interferon, is administered in such a way that sufficient doses of γ-interferon are supplied. These doses depend

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notably on the administration procedure, the body weight, and the nature and the severity of the disease to be treated. For example, by the subcutaneous or intramuscular route, one can administer 10^5-10^7 units of γ-interferon by injection, or administer 3-7 injections per week.

The drug of the invention can be used in all the cases where one of the activities of γ-interferon is of therapeutic advantage. These activities include the immunostimulant effects, for example, the antiproliferative effect in cancers, and the activation of the immune defenses in infectious diseases (viral, bacterial or parasitic), or the capacity to block the synthesis of collagen in fibrosis of organs, etc.

Thus, the invention also relates to an improved method for treating with γ-interferon, in which one administers, besides γ-interferon, a modulating agent as defined above. The modulating agent can be administered at the same time as the γ-interferon (for example, by intravenous administration) a short time (notably less than 2 min) before or after the γ-interferon.

γ-Interferon is generally presented in the form of a lyophilizate which one dissolves in a sterile liquid vehicle at the time of use. The potentiating agent of the invention can be presented in the form of a lyophilizate, or in the form of an aqueous solution (particularly a solution in physiological serum), and, in this last case, one can solve the lyophilized γ-interferon in the solution of modulating agent.

The invention also concerns a method for the modulation of the activity of γ-interferon, which is intended notably to allow an action of γ-interferon by the systemic, and not only local, route, and/or intended to increase the half life of γ-interferon in the organism, to prevent its degradation in the organism and to promote its activation and/or to modulate the activity, notably the local activity, of endogenous γ-interferon, where this method is characterized in that one administers to a subject in need of such a drug, a drug comprising as active ingredient a modulating agent as defined above. When the drug is used in combination with exogenous γ-interferon, one administers the potentiating agent as defined above at the same time as γ-interferon, or a short time before or after.

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The following examples illustrate the invention.

Example I: Modulating agent derived from heparane sulfate

1: Obtention of tritium-labeled heparane sulfate

One cultures fibroblasts from human skin until confluence, then the cultures are incubated for 72 h in

a medium DMEM/10% FCS containing 10 μCi/mL (or 0.7 MBq/mL) tritiated glucosamine

(Amersham). After 72 h of incubation, one separates the cells from the culture medium and one subjects

them to the action of trypsin for 15 min, then one centrifuges and collects the supernatant which after the

addition of culture medium to it, is loaded on a column of DEAE Sephacel (Pharmacia) equilibrated

with a buffer of 20mM sodium phosphate 0.15M NaCl, pH 6.8. One applies a linear gradient of sodium

chloride 0.15-0.8M at 0.2 mL/min. One collects the peak corresponding to the proteoglycans heparane

sulfate, which is eluted with approximately 0.5-0.6M NaCl. After dialysis against distilled water, to

eliminate the salts, one evaporates the solvent under a vacuum and one subjects the residue to a

digestion with chondroitinase ABC (Sigma) and papain (Sigma) to eliminate the chondroitin sulfates

that may contaminate the preparation, and to degrade the protein part of the proteoglycans with heparane

sulfates.

One subjects the product obtained to chromatography on a column of DEAE Sephacel and one

collects the purified heparane sulfate, which is eluted with a buffer of sodium phosphate 20mM, NaCl

1M.

DMEM stands for: Dulbecco modified Eagle medium.

FCS stands for: fetal calf serum

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2: Preparation of an affinity chromatography column (ligand: γ-interferon)

One mixes 1 mL of chromatography gel Affi-Gel 10 (BioRad) with 1 mL buffer 5mM tris/HCl at pH 6.8, containing 0.5 mg human γ-interferon (ROUSSEL-UCLAF) and 1 mg heparin (Sigma). It is known that the gel Affi-Gel comprises reactive groupings that allow the fixation of the amine groups of proteins. The presence of an excess amount of heparin in the coupling buffer is intended to protect the C-terminal region of the γ-interferon which otherwise could react with the matrix of Affi-Gel. After 16 h of contact at 4°C with stirring, one loads the product obtained on a column, and one washes it with a buffer 50mM tris/HCl at pH 6.8, then with the same buffer containing additionally 2M NaCl. Before the utilization, the affinity chromatography column obtained is washed with a solution of 2M NaCl then equilibrated with a buffer of 10mM tris/HCl at pH 6.8 (equilibration buffer). For the affinity chromatography, one uses 200-μL samples in the equilibration buffer, and one elutes with a gradient of sodium chloride from 0 to 1M.

On the affinity chromatography column so obtained, the heparin binds and it is eluted by solutions of sodium chloride having a concentration of more than approximately 0.5M. Similar results are obtained consistently when the experiment is repeated 20 consecutive times.

3: Fractionation of heparin sulfate and of its depolymerization products on the γ-interferon affinity matrix

The tritium-labeled heparin sulfate obtained above was applied to the γ-interferon affinity column, then one elutes successively with solutions of sodium chloride with increasing concentrations: 0.1-0.2-0.3-.....-1M. One observes that the heparane sulfate binds to the interferon of the affinity column, and can be eluted in a fractionated way with solutions in the range 0.3-0.6M NaCl.

The depolymerization of heparane sulfate (HS) with nitrous acid and with heparinase (Sigma) produces residues that have lost practically all their affinity for γ-interferon.

The depolymerization of HS by heparitinase (Sigma; 10 U/mL) yields 60-70% products that do not bind with interferon; the rest of the products obtained bind to interferon and are eluted with solutions in the range 0.1-0.3M NaCl. The affinity for interferon of these last products is thus decreased, in comparison to an intact HS.

The product of the digestion by heparitinase, which bind with interferon, are N-sulfated oligosaccharides, which have polymerization degrees of 2-12 and can be separated by a molecular mesh on a column of Bio-Gel P-6 (BioRad). The fractions obtained are loaded individually on the γ-interferon affinity column. The affinity increases with the degree of polymerization of the oligosaccharides, but it is relatively low, since the longest oligosaccharides (degree of polymerization: 12) have already been eluted with the concentrations of sodium chloride from 0.2 to 0.3M.

4: Digestion of heparane-sulfate protected by γ-interferon

In a buffer solution 50mM tris/HCl, pH 7.6 containing 10μM γ-interferon (concentration calculated on the basis of the monomer molecule) and approximately 5μM heparane sulfate, one adds heparitinase to a final concentration of 25 U/mL.

In addition, one carries out a digestion of HS under the same conditions but in the absence of γ-interferon for comparison.

The products of the digestion (4 h at 30°C) were analyzed by chromatography on Bio-Gel P-10. One elutes with a solution of 0.5M ammonium hydrogen carbonate at 4 mL/h. One observes that the digestion of HS by heparitinase supplies oligosaccharides having a degree of polymerization (dp) of 2-14.

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The digestion products (4 h at 30°C) were analyzed by chromatography on Bio-Gel P-10. One elutes with 0.5M solution of ammonium hydrogen carbonate at 4 mL/h. One observes that the digestion of HS by heparitinase has produced oligosaccharides with a degree of polymerization (dp) of 2-14.

When the digestion is carried out in the presence of γ-interferon, one observes at the [sic] elution of supplemental peaks. The peaks obtained are collected, lyophilized and resuspended in the equilibration buffer used for the affinity chromatography. One observes thus the first supplemental peak (peak I) as an affinity which is practically identical to that of intact HS. The products of peak I are eluted, in the affinity chromatography column with solutions at concentrations of approximately 0.4 and 0.5M NaCl (majority products) and minority products are eluted at concentrations of approximately 0.3 and 0.6M NaCl.

Similar experiments, repeated with heparane sulfates from fibroblasts originating from two different donors produced identical results.

The products of peak I thus correspond to a domain of HS which is protected by γ-interferon notably against depolymerization by heparitinase. These products are called "IPD" hereafter.

IPD is thus a fragment of heparin sulfate which has a strong affinity for the γ-interferon with which it is capable of binding. The IPD is characterized further below.

5: Characterization of IPD

The IPD was subjected to depolymerization reactions under the following conditions:

a) depolymerization with nitrous acid, at pH 1.5 for 15 min at ambient temperature; and

b) depolymerization with heparinase (10 U/mL) for 15 h at 30°C.

In each case, the depolymerization of the IPD was compared with the depolymerization of the HS from which it originated.

The depolymerization products were compared by sieving through a molecular mesh on Bio-Gel P-6.

One observes that one obtains, with IPD, 35% disaccharides compared to 18% only for HS, indicating a higher number of contiguous disaccharides, whose glucosamine is N-sulfated, in the IPD than in HS. Moreover, the size of the HNO_2-resistant oligosaccharides is smaller in IPD than in HS, indicating the absence of long sequences without N-sulfated glucosamine in IPD. The analysis of the digestion profiles of IPD and HS makes it possible to calculate it (using the formula F = sum of H_i/n_i, where H_i is the percentage of radioactivity in peak No. i, and n_i is the number of disaccharides of this peak) that 61% of the disaccharides of IPD are N-sulfated, compared to only 42% for HS. In addition, the distribution of these disaccharides is different in IPD and in HS, as mentioned above. Finally, the /1· superior sensitivity of IPD to the action of heparinase (compared to HS) demonstrates that the IPD is enriched with disaccharides containing an iduronic acid sulfated in position 2.

Moreover, the IPD contains a domain which is resistant to heparitinase. The molecular weight of this domain is determined and compared to that of IPD by molecular mesh [sieving] on Sepharose CL-6B (Pharmacia). The approximate molecular weights of the samples were determined according to the method of Wasteson, J. Chromatogr. 59, 87-97 (1971). It has been found that IPD, on the one hand, and its heparinase-resistant domain, on the other hand, have molecular weights of 10 and 7 kDa, respectively.

IPD and its heparinase-resistant domain (HR-IPD) have been subjected to a depolymerization by heparitinase under the following conditions: buffer 50mM tris HCl, pH 7.6 for 15 h at 35°C, in the presence of 10 U/mL heparitinase.

It is recalled that one unit (U) of heparitinase is the quantity of heparitinase that allows the production, by reacting with heparane sulfate, of 0.1 µmol unsaturated uronic acid per hour.

The depolymerization products were analyzed on a column of Bio-Gel P-6.

The HR-IPD domain is very sensitive to the depolymerization by heparitinase, which yields 67% disaccharides and 26% tetrasaccharides. The analysis of the digestion profile shows that 82% of the bonds between disaccharides have been cleaved. This shows that this region is rich in disaccharides containing a glucuronic unit, the majority of these disaccharides being N-acetylated.

The analysis of the composition of the disaccharides obtained by depolymerization of HR-IPD with heparitinase was carried out by HPLC on an anion exchange column. This analysis shows a high content of nonsulfated N-acetylated disaccharides (58%) and 31% of N-sulfated disaccharides without O-sulfate groups. In addition, the treatment of the intact IPD with heparitinase yields two principal heparitinase-resistant products corresponding to hexasaccharides and octasaccharides that are not present in the HR-IPD domain. These fragments thus represent the highly N-sulfated sequences which are cleaved and partially degraded by the treatment with heparinase. These fragments are completely degraded to disaccharides by nitrous acid, which shows that they consist of contiguous sequences of N-sulfated disaccharides. These fragments are also cleaved by heparinase, the octasaccharides yielding primarily tetrasaccharide products, and the hexasaccharides yielding a mixture of tetrasaccharides and disaccharides, which indicates the presence of N-sulfated (optionally 6-sulfated) glucosamine-2-sulfated iduronic acid units.

The affinity of these hexasaccharides and octasaccharides for the interferon was also tested with the help of the affinity chromatography column. It was observed that these oligosaccharides have only a weak affinity for γ-interferon, similar to the one observed for the fragments of the same size obtained by reacting heparitinase with intact HS (see 3 above).

Similarly, the HR-IPD domain has only a weak affinity for γ-interferon and it is eluted with concentrations of sodium chloride of 0.1-0.2M.

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Thus, the IPD comprises regions of distinct structure which, although individually presenting only a weak affinity for γ-interferon, act synergistically as far as the affinity for γ-interferon is concerned, after they are combined.

6: Crosslinking of γ-interferon by heparane sulfate

One mixes 250 ng γ-interferon with different concentrations of HS in an aqueous solution, and one incubates for 30 min at 4°C with 2mM EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimine), a hetero-bifunctional coupling agent (marketed by Pierce) in the presence of N-hydroxysulfosuccinimide (S-NHS), which activates the carboxylic groups, at a concentration of 1mM, to increase the coupling yield of EDC. After 15 min, one stops the reactions with the help of a buffer called electrophoresis sample, and one analyzes the crosslinkage products by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS-PAGE). It is recalled that a so-called electrophoresis sample buffer contains approximately 2% sodium dodecyl sulfate which denatures the proteins (Laemmli, Nature, 227, 680-685 (1970).

Moreover, the γ-interferon was reacted with a bis(sulfosuccinimidyl) suberate (abbreviated BS³), a homo-bifunctional coupling agent marketed by Pierce.

It has been found that γ-interferon migrates like a 17-kDa monomer by electrophoresis in a /1
denaturing medium (gel of 15% polyacrylamide, 0.1% SDS, 4M urea).

When the γ-interferon has reacted with BS³ before the electrophoresis, one observes a 34 kDa band, which shows that the molecule was in the form of a dimer.

In contrast, the reagent EDC/S-NHS does not crosslink the two monomers of γ-interferon, probably because there is no amino group in a favorable position in proximity to a carboxylic group between the two monomers.

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When γ-interferon is reacted with the system EDC/S-NHS in the presence of various concentrations of HS originating from the bovine intestinal mucosa (Sigma) to crosslink the carboxylic groups of uronic acid of HS with the aminated groups of γ-interferon, the material obtained has a molecular weight of approximately 50 kDa with two minor bands at 17 and 34 kDa. This molecular weight of 50 kDa is compatible with the one expected for a dimer of γ-interferon (34 kDa) crosslinked with a molecule of HS (16 kDa). Even if one uses a large excess of HS (10 mol per 2 mol of monomer γ-interferon), one always obtains the product having a molecular weight of 50 kDa, which shows that it is a molecule of HS bound to 2 monomers of γ-interferon (34 kDa).

The result of the preceding experiments is that the IPD domain of HS consists of an internal domain of 7 kDa, each end of which is connected with a terminal hexa- and/or octa-saccharide N-sulfated oligosaccharide, where the internal domain contains predominantly N-acetylated disaccharides that are rich in glucuronic acid. Taking into account its molecular weight, the internal domain contains approximately 15-16 disaccharide units.

Example II: Modulating agent derived from heparin, dextran sulfate, or fucoidan

Any molecule (derived from heparin or other anionic polymers) capable of binding by affinity with the two C-terminal ends of γ-interferon dimer, to mask the sites of interaction with heparane sulfate, can be used as a γ-interferon modulating agent. The search for such molecules can be carried out using a competition study. For example, one can evaluate the capacity of a molecule to compete for the binding to γ-interferon with the radio-labeled intact heparane sulfate (or the IPD). These molecules can be heparin (optionally partially desulfated to decrease its anticoagulating activity), dextran sulfate (a glucose polymer containing up to 3 sulfate groups per glucose residue and having a reduced anticoagulating activity - 17 international units heparin per mg; Ricketts, W. Biochem. J., 51, 129

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(1952)), fucoidan (a polysaccharide consisting of sulfated fucoses: see Black, W.A.P. et al., J. Sci. Food Agri., 3, 122 (1952) and Grauffel, V. et al., Biomaterials, 10, 363 (1989)), etc.

Such a study is illustrated here with heparin molecules of different molecular weights (1.8 - 4.5 - 9 - 12.5 - 21 kDa). γ-Interferon (0.3µM) is incubated in 100 µL of buffer 10mM tris/HCl, pH 6.8, for one hour at ambient temperature, with 10,000 cpm heparane sulfate and with heparane molecules whose concentrations one can adjust. The reaction mixtures are then aspired through a nitrocellulose filter (porosity 0.45 µm) with the help of a "dot-blot" system connected to a vacuum pump. The nitrocellulose filters are then rinsed two times with 200 µL buffer 10mM tris/HCl, pH 6.8, then dissolved individually with 500 µL DMSO and subjected to a liquid scintillation count. In this experiment, the γ-interferon is adsorbed to the nitrocellulose and it retains with it molecules that are associated with it. The molecules (heparane sulfate or heparin) that have remained free traverse the nitrocellulose filter without being retained. This test is based on the strong affinity of nitrocellulose for proteins, and on its absence of interaction with polysaccharides. In the absence of a competitor, the nitrocellulose filter retains approximately 3000 cpm heparane sulfate. In the presence of heparin, the quantity of heparane sulfate bound to γ-interferon decreases, demonstrating that heparin is competing with heparane sulfate to bind with γ-interferon. This experiment has also shown that the molecules or fragments of heparin must have a molecular weight of at least 9 kDa to interact effectively with γ-interferon.

In the following experiments, the modulating effect of γ-interferon was studied.

a) The potentiating effect increases the half life of γ-interferon in the circulation   /1:

Human γ-interferon, radio-labeled with iodine 125, was injected into rats, by the intravenous route, in the amount of 40 µg/kg body weight, alone or in combination with heparin (2000 U/kg body weight) as

potentiating agent. The circulating level of γ-interferon was monitored over time by measuring the radioactivity that could be precipitated with 10% TCA, in the plasma fractions.

Injected alone, γ-interferon is eliminated from the blood flow, exponentially. Approximately 90% of the cytokine disappears during the first 5-10 min with a half life ($t_{1/2}$) of 1.1 min. The remaining 10% (2nd part of the exponential curve) are eliminated much more slowly, with a $t_{1/2}$ of 94 min. On the other hand, when γ-interferon is coinjected with heparin as potentiating agent, its plasma concentration describes a monoexponential curve characterized by a half life of 99 min.

It should be noted that the receptor of γ-interferon does not participate in the observed effects, because the human γ-interferon used here is not recognized by the interceptor of γ-interferon from murine species. In addition, an injection of heparin a few minutes after the injection of γ-interferon alone puts the cytokine back into circulation as a result of the competitive effect with endogenous molecules of the heparin type.

b) The potentiating agent increases the in vivo biological activity of γ-interferon

Human γ-interferon (4 x 10^5 U) was injected by the intravenous route into rats weighing approximately 500 g, in combination or not with heparin (2000 U/kg body weight) as potentiating agent. At the times 2, 10, 60 and 120 min after the injection, plasma samples were collected to perform a measurement of the γ-interferon activity.

The biological activity of γ-interferon is measured as follows. A confluent layer of Wish cells (Hayflick, L., Exp. Cell Res. 23, 14-20 (1961)) is placed in the presence of a standard γ-interferon concentration range (generally from 100 to 0 U/mL, with 2 by 2 dilutions) or of sample to be assayed. After 24 h of incubation in a CO_2 oven at 37°C, the cells are infected with 100 μL VSV (vesicular stomatitis virus), whose concentration is adjusted so as to produce total lysis of the cells in 24 h, in the

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absence of γ-interferon. In this test, the dose of γ-interferon which protects 50% of the cells from lysis by VSV corresponds to 1 U/mL. The measurement of the proportion of protected cells can be carried out according to the method described by Mosmann, T., J. Immunol. Meth. 65, 55-63 (1983).

After the injection of γ-interferon alone, 42%, 3.7% and 1.9% of the activity injected at times 2, 60 and 120 min, respectively, were found, whereas 7.4% and 5.7% of the quantity injected are still present in the plasma at times 60 and 120 min, respectively. In the absence of a potentiating agent, γ-interferon is thus partially inactivated.

In the presence of heparin used as potentiating agent, 2 min after the injection an activity was measured which corresponds to 600% of that that had been injected. Consequently, there was a very rapid increase in the specific activity of γ-interferon in the presence of the potentiating agent. Two hours after the injection, the γ-interferon is still twice as active as before the injection. If one combines the increase in the activity and the increase in the quantity of circulating γ-interferon, there is approximately 50 times more interferon activity in the circulating blood two hours after the injection, thanks to the potentiating agent. The injection of heparin alone does not cause the appearance of any interferon type activity in the plasma of animals.

Similar results, as far as the lifetimes and activity increase are concerned, can be observed by replacing the heparin either with heparin fragments having a molecular weight of 10 kDa, or with the IPD fragment described in Example I above, or with dextran sulfate or fucoidan.

Claims

1. Utilization as active ingredient in the preparation of a γ-interferon activity modulating drug of a modulating agent comprising a grouping of formula A-X-B, in which A and B represent independently an oligosaccharide group carrying a sufficient anionic charge to confer to said oligosaccharide group an affinity for a part of the C-terminal end of human γ-interferon containing the peptide sequence 125-131, and X is a spacer arm of sufficient length to allow the groups A and B to bind each with one of said peptide sequences of the C-terminal ends of a homodimer of γ-interferon.

2. Utilization according to Claim 1, characterized by the fact that said oligosaccharide groups carries sulfate and/or phosphate groups.

3. Utilization according to Claim 1 or 2, characterized by the fact that said oligosaccharide group contains units derived from an N-sulfated osamine.

4. Utilization according to Claim 3, characterized by the fact that said oligosaccharide group contains alternating units derived from said osamine and units derived from an optionally sulfated uronic acid.

5. Utilization according to any one of the preceding claims, characterized by the fact that said oligosaccharide groups are depolymerization fragments that can be obtained by reacting heparitinase with heparane sulfate.

6. Utilization according to any one of the preceding claims, characterized by the fact that said spacer arm is a polymer containing anionic groups.

7. Utilization according to the preceding claim, characterized by the fact that said anionic groupings are chosen from the sulfate, phosphate and carboxylic groupings.

8. Utilization according to any one of the preceding claims, characterized by the fact that said spacer arm is derived from a polyoside, a polyglycol, a poly(glycolic acid) or a poly(lactic acid).

9. Utilization according to any one of the preceding claims, characterized by the fact that said spacer   /2

arm is derived from a polyoside chosen from polydextran, cellulose, starch, carrageenan, fucoidan and a glycosaminoglycan, and their derivatives.

10. Utilization according to any one of the preceding claims, characterized by the fact that said spacer arm is a depolymerization fragment obtained by reacting heparinase with heparane sulfate.

11. Utilization according to any one of Claims 1 and 2, characterized by the fact that said compound is an incompatible polymer containing anionic groupings in a sufficient quantity at at least two sites corresponding to A and B, as defined in Claim 1.

12. Utilization according to the preceding claim, characterized by the fact that said compound is a dextran sulfate or fucoidan.

13. Utilization according to any one of the preceding claims, characterized by the fact that said drug is free of γ-interferon.

14. Utilization according to any one of Claims 1-12, characterized by the fact that said drug contains γ-interferon.

15. Drug based on γ-interferon, characterized by the fact that it contains a modulating agent as defined in any one of the preceding claims.

16. Drug according to the preceding claim, characterized by the fact that it contains approximately one mole modulating agent per two moles γ-interferon.